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Introduction

Malignant transformation and progression of human cancer is frequently associated with overabundance of proteins that are involved in normal cellular processes, such as proliferation, differentiation, migration or apoptosis. Examples of such proteins include members of human epidermal growth factor receptor (hEGFR) and cellular Src (c-Src) tyrosine kinase families, which are frequently cooverexpressed in human neoplasms and especially in breast cancer. Results from recent studies using cultured fibroblasts and human breast cancer cell lines have indicated that c-Src and EGFR synergistically interact to promote tumor formation in nude mice xenografts. To check whether this synergism occurs in the more physiological setting of the mammary gland, I am going to test the interaction of these tyrosine kinases in a transgenic mouse model, where MMTV-c-Src and MMTV EGFR transgenic mice will be generated, cross-bred to form bigenic mice, then examinated for tumor formation in mammary gland tissue. If the synergism hypothesis is correct, bigenic mice should develop large tumors more rapidly than single transgenic mice, thus validating the synergism between c-Src and EGFR as a target for future therapies.

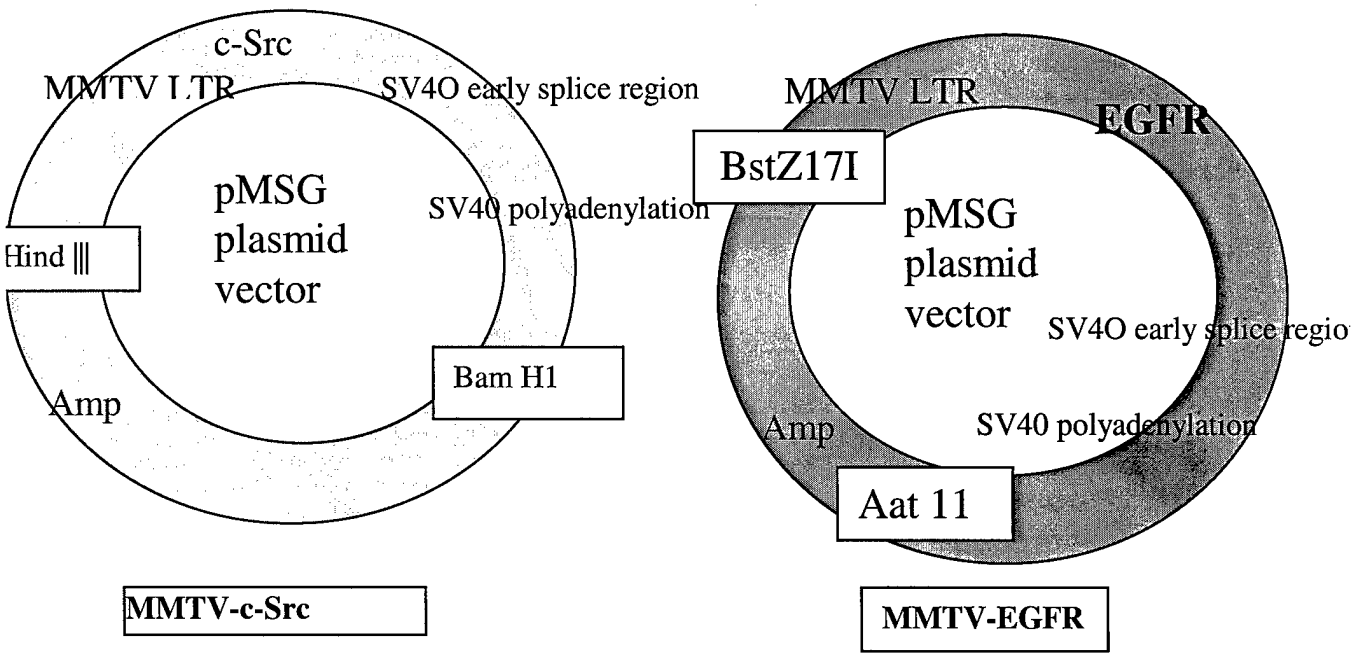
Annual summary for Postdoctoral Fellowship Award BC 020517 " The Impact of Tyrosine Kinase Signaling on Breast Cancer Development" on behalf of N.V. Marozkina.

Ī. Research accomplishments associated with the tasks outlined in the approved Statement of work.

1. To construct MMTV-c-Src and MMTV-EGFR transgenes and test their expression in cell culture (month 1-6)

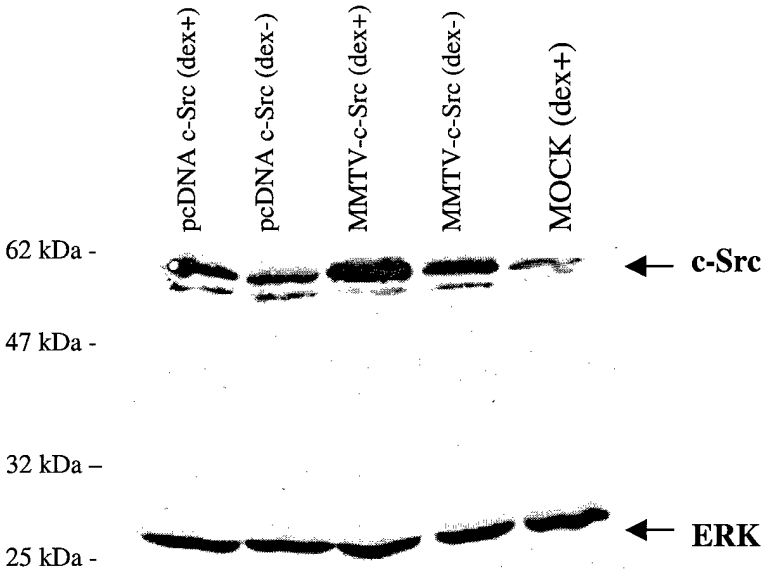
a) Insert cDNA encoding human MMTV-c-Src gene into the pMSG vector
b) Insert cDNA encoding human MMTV-EGFR gene into the pMSG vector

Our choice of a hormone-responsive, inducible vector system to construct the transgenes in is the pMSG vector (Pharmacia Biotech), which drives expression of the cDNA of choice from the MMTV promoter. The MMTV promoter responds transcriptionally to glucocorticoid and steroid receptors, such as estrogen and androgen receptors, which are expressed in the female and male reproductive organs, respectively. I have inserted the cDNA encoding human MMTV-EGFR or chicken MMTV-c-Src into this vector, using established recombinant DNA techniques. I have accomplished this for both cDNAs.

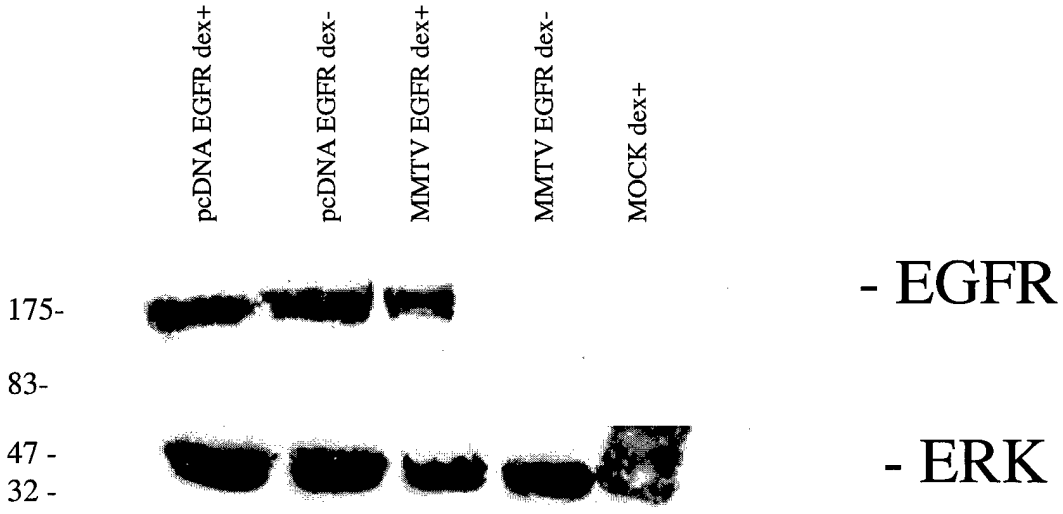


c) Test the expression of EGFR and c-Src constructions in MCF7 breast cancer cells

Then I have tested the inducible expression of these constructions in MCF7 breast cancer cells, which endogenously express estrogen receptors that bind estrogen or dexamethasone and become transcriptionally active.



Dexamethasone inducible expression of c-Src from the MMTV transgene in MCF7 breast cancer cells.



Dexamethasone inducible expression of MMTV-EGFR in MCF7 breast cancer cells.

d) Perform PCR reactions in order to detect the presence of c-Src or EGFR cDNA against a background of total mouse genomic DNA. Sensitivity should be one copy of cDNA/genome.

As a control for the detection of the presence of a chicken c-Src cDNA or a human EGFR cDNA, each stably integrated into mouse genomic DNA, PCR was performed using chicken c-Src or human EGFR primers and genomic DNA from mouse fibroblast 10T1/2 cell lines that have an integrated copy of chicken c-Src cDNA or human EGFR cDNA.

MMTV-human EGFR primers

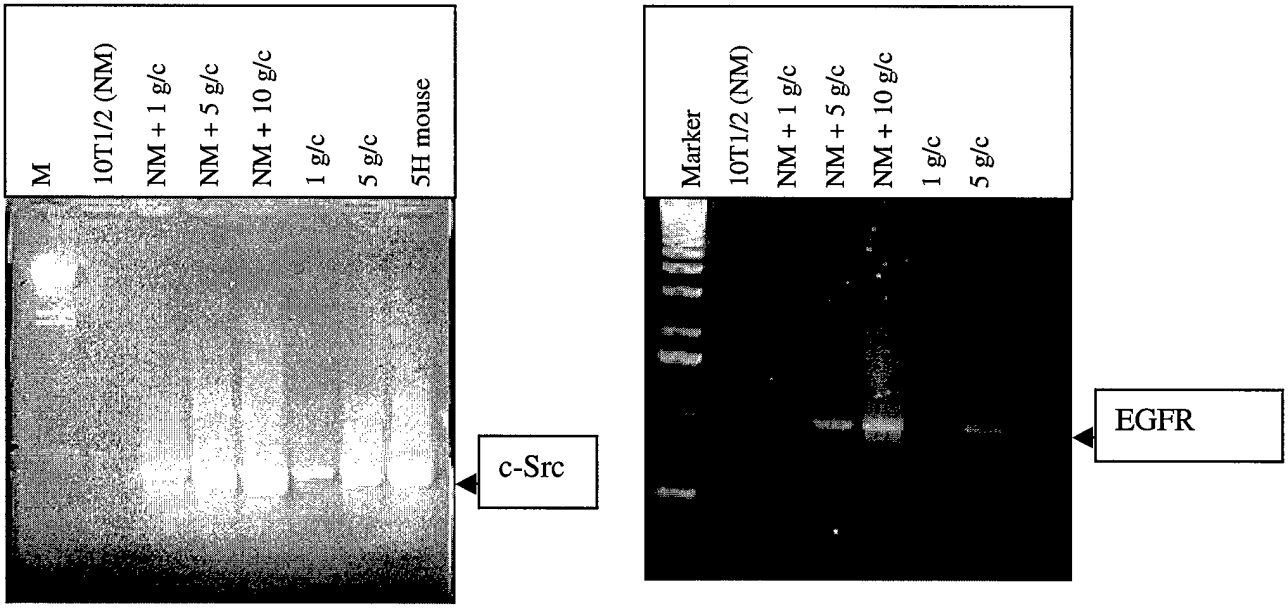
5' - GAT CGG CCT CTT CAT CGG – 3'
5' - TTC TTT CAT CCC CCT GAA TG – 3'

MMTV-chicken c-Src primers

5' – ACCCCCAACAAGACAGCAGCC – 3'
5' – CAAAGTCAGAAACGGAGAGGC –3'

Chicken-specific primers for c-Src and human-specific primers for EGFR were used for PCR reactions to detect the presence of the transgenes, against a mouse background. The MMTV-c-Src primers gave a PCR product of about 0.587 kb and encompassed base pairs 99 through 686. The MMTV- EGFR primers gave a PCR product of about 1 kb and encompassed base pairs 2174 through 3146.

I used genomic DNA from 2 cell lines as templates – 10T1/2 and 5H mouse fibroblasts. 10T1/2 – are parental mouse fibroblasts expressing endogenous mouse c-Src and EGFR; 5H are 10T1/2 cells stably expressing chicken c-Src.



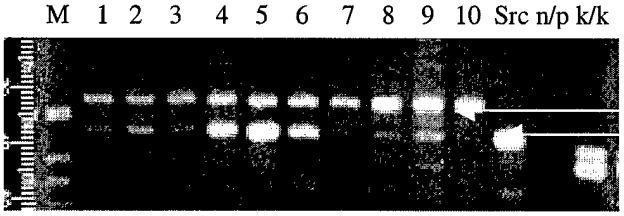
PCR shows the presence of a chicken c-Src cDNA in genomic DNA derived from the 5H mouse fibroblast cell line, but not from the normal mouse (NM) 10T1/2 parental line. I detected the presence of chicken c-Src only in the 5H cells, which shows the specificity of the reaction and as little as 1 gene copy. 1 gene copy is intended to create control template preparations, in which tail DNA is spiked with known amounts of transgene DNA to create single copy and multiple copy standards. These are used to verify that Southern blot or PCR reaction is sensitive enough to detect the integration of a single copy of a transgene into mice genome. This will ensure that the screen for transgenic founders will not miss any transgenic lines of mice.

Quantitative amplification of the plasmid DNA (MMTV-EGFR) was detected as well as specificity of the reaction (absence of product amplification in normal mouse DNA, 10T1/2) using these primers.

Task 2. To generate and maintain MMTV-c-Src and MMTV-EGFR transgenic mice

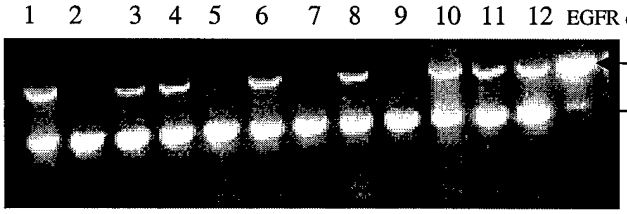
a) Perform blastocyst injection and implantation of embryos into pseudopregnant females
b) Test the genomic DNA from tail crops of pups by PCR for the presence of transgenes

The Transgenic Mice Core Facility of the University of Virginia assisted me in all aspects of this phase of the project. I provided the facility with the expression vector, and they performed blastocyst injection and implantation of embryos into pseudopregnant females. I tested genomic DNA from tail crops by PCR and Southern blotting for the presence of transgenes.



1-10 samples of transgenic mice genomic DNA
Src- plasmid DNA, positive control
N/p –negative control, non-transgenic mice genomic DNA
K/k- kit control
SNO- internal control for mice genomic DNA

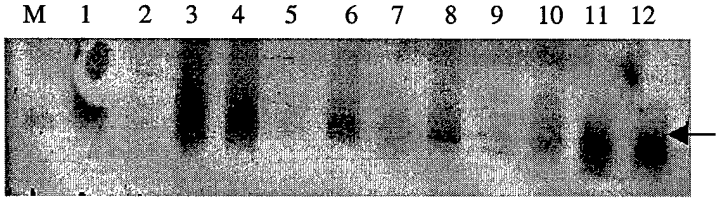
MMTV-c-Src transgenic mice. PCR.



1-12 samples of transgenic mice genomic DNA
EGFR- plasmid DNA, positive control
OMO1/2- internal control for mice genomic DNA

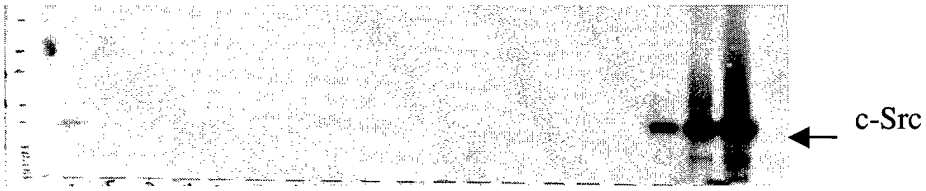
MMTV-EGFR transgenic mice . PCR.

c) Confirm the presence of transgenes and estimate the number of transgenes that got integrated per genome by Southern blot analysis.



1-12 samples of transgenic mice genomic DNA

MMTV-EGFR transgenic mice. Southern blot analysis

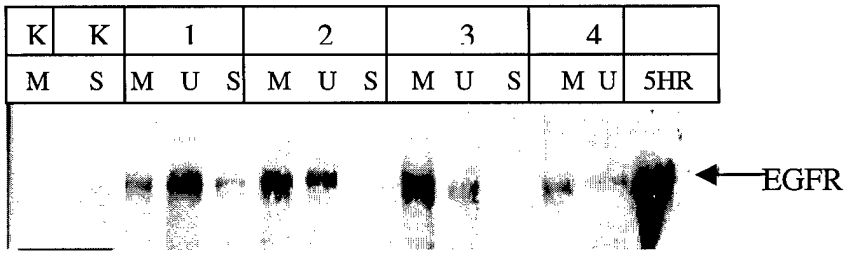


1-16 samples of transgenic mice genomic DNA

MMTV-c-Src transgenic mice. Southern blot analysis

Mouse (sample 1 on this figure) that was positive by Southern blot did not give any positive pups. It did not transmit transgene into the next generation.

d) On animals that score positively for the transgene, assess the protein overexpression by immunohistochemistry of paraffin-embedded tissue section and Western blotting of excised and solubilized mammary gland tissue.



M- mammary gland
U- uterus
S-saliva
K-nontransgenic mouse control
1-4 samples of transgenic mouse tissues.
5HR – mouse fibroblast cell line that stably expresses human EGFR.

MMTV-EGFR transgenic mice. Western blot analysis of immunoprecipitated human EGFR.

e) Continue the transgenic line of mice by breeding either with non-transgenic or transgenic litter-mates.

III. Technical and unexpected difficulties.

MMTV c-Src transgenic mice did not give any positive pups. None of additional microinjections were successful either. Probably c-Src did not integrate into germ line and animals were mosaic. It is also possible that the c-Src transgene could be toxic to mice genome.

Several approaches to overcome this problem:

1. I can breed MMTV-EGFR with MMTV-TGF-α transgenic mice to see the increased tumor formation in MMTV-EGFR/ TGF-α bigenic transgenic mice.
2. I can obtain constitutively active c-Src^{527} mice and breed them with the MMTV-EGFR transgenic mice.
3. I can make a new MMTV c-Src plasmid construction, using other (BSL1- STOP VENUS) vector. BSL1- STOPVENUS-c-Src will be expressed in all tissues of mice. Then I will breed mice mentioned above with MMTV-Cre transgenic mice to get MMTV c-Src transgenic mice with c-Src targeted expression in mammary glands.

I plan to pursue all three approaches.

Key research accomplishments.

1. MMTV-c-Src and MMTV-EGFR transgenes have been constructed in plasmid vectors and their expression was tested in cell culture.

2. PCR reactions were performed in order to detect the presence of c-Src or EGFR cDNA against a background of total mouse genomic DNA.

3. MMTV-c-Src and MMTV-EGFR transgenic mice were generated. Presence of transgenes was confirmed by PCR and Southern Blot.

4. On animals that scored positively for the EGFR transgene, protein overexpression was assessed by Western blotting of excised and solubilized mammary gland, uterus and salivary gland tissues.

5. The results of transgenenic mice generation were presented at the scientific conference in Keystone, Colorado "Mouse model of human cancer" – "Role of epidermal growth factor in tumorigenesis: transgenic mice generation". Abstract is enclosed.

Reportable outcomes

1. List of reportable outcomes that have resulted from this award to include:

    a) Abstract from the conference in Keystone, Colorado "Mouse model of human cancer" – "Role of epidermal growth factor in tumorigenesis: transgenic mice generation".

Abstract is enclosed. See appendices.

Conclusions.

1. MMTV-c-Src and MMTV-EGFR transgenic mice were generated. Presence of transgenes was confirmed by PCR and Southern Blot.

2. On animals that scored positively for the EGFR transgene, protein overexpression was assessed by Western blotting of excised and solubilized mammary gland, uterus and salivary gland tissues.

3. At the present time, none of the EGFR transgenic mice that we have developed have visible tumors; however, they will be examined for evidence of displasia, particularly in steroid-responsive tissues, such as mammary gland, uterus, ovary and prostate.

4. Interaction with other signaling molecules such as growth factors, intracellular transducers, or nuclear transcription factors may play a role in EGFR-induced tumorgenesis. To determine this, MMTV-EGFR transgenic mice will be bred with other transgenic mice such as those expressing c-Src or TGF-α, to assess tumor formation. It is expected that breast tumors will form in these bigenic mice, providing evidence for the role of EGFR in tumorigenesis.

Role of epidermal growth factor in tumorigenesis: transgenic mice generation.

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Key words: epidermal growth factor receptor, transgenic mice, tumorigenesis

The human epidermal growth factor receptor (hEGFR, HER 1) is one of a family of receptor tyrosine kinases (RTK) that consists of four known members (EGFR/ HER1/erbB1, HER2/neu/ erbB2, HER3/erbB3, HER4/erbB4). Upon binding of ligand, receptors dimerize, are catalytically activated, and trans – phosphorylate their partners on carboxyl-terminal tyrosine residues, which in turn act as docking sites for multiple signaling proteins. These molecules induce changes in gene expression, which bring alterations in morphogenesis, mitogenesis and motility. Members of HER family (including EGFR) are overexpressed in a wide range of human tumors including the brain, lung, breast, stomach, liver, prostate, ovary and bladder. Much evidence suggests that EGFR is involved in the later stages of human breast cancer and may play a role in growth and metastatic processes. EGFR also plays a variety of roles in normal tissue development and is found in ductal epithelial cells of normal breast tissue. According to the data in tissue culture, EGFR itself is a weak oncogene. In order to test the role of EGFR overexpression in breast tumor development, MMTV-EGFR transgenic mice have been developed using the hormone responsive inducible vector system based on the MMTV promoter. This promoter responds transcriptionally to glucocorticoids and steroids. At the present time, none of the EGFR transgenic mice that we have developed have visible tumors; however, they will be examined for evidence of displasia, particularly in steroid-responsive tissues, such as mammary gland, uterus, ovary and prostate. Interaction with other signaling molecules such as growth factors, intracellular transducers, or nuclear transcription factors may play a role in EGFR-induced tumorgenesis. To determine this, MMTV-EGFR transgenic mice will be bred with other transgenic mice such as those expressing c-Src or TGF-α, to assess tumor formation. It is expected that breast tumors will form in these bigenic mice, providing evidence for the role of EGFR in tumorigenesis.